

Investigation of the Physiological Responses of Belugas to “Stressors” to Aid in Assessing the Impact of Environmental and Anthropogenic Challenges on Health

Tracy Romano (PhD) & Tracey Spoon (PhD)
Mystic Aquarium, a division of Sea Research Foundation
55 Coogan Blvd.
Mystic, CT 06355

phone: (860) 572-5955 ext. 102 fax: (860) 572-5969 email: tromano@mysticaquarium.org
phone: (860) 572-5955 ext. 139 fax: (860) 572-5969 email: tspoon@mysticaquarium.org

Stephen Lamb
Animal Health Diagnostic Center Endocrinology
College of Veterinary Medicine
Cornell University
Ithaca, NY 14853
phone: (607) 253-3593 fax: (607) 253-3943 email: svl2@cornell.edu

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LONG-TERM GOALS

The overall top level goal of this effort is to investigate the physiological i.e. neuroimmunoendocrinological responses of beluga whales to “stressors”. “Stressor events” will allow for a better understanding and characterization of the relationships among hormones (e.g. cortisol, corticosterone, adrenocorticotropin hormone, aldosterone, catecholamines) in different matrices (blood, saliva, blow, feces) in conjunction with immune function. In addition, “stressor events” will enable us to define and compare the quantitative and temporal relationships of hormones across the different matrices.

OBJECTIVES

The objectives of this effort are: 1) To monitor the neuroimmunoendocrinological responses (via saliva and blood) of three resident aquarium belugas before and after the introduction and throughout the adaptation process of seven new belugas to their habitat and to measure the neuroimmunoendocrinological responses of 5 belugas to transport and 2) To monitor the neuroimmunoendocrinological responses (via blood, saliva, blow and feces) of 2-3 aquarium resident whales before and after the occurrence of a known stressor.

APPROACH

Seven belugas, *Delphinapterus leucas* (one male 22 years of age, four females 9-26 years of age, two calves- 1 male and 1 female < 2yrs) were transported from Shedd Aquarium, Chicago, IL to Mystic

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Aquarium, Mystic, CT in the fall of 2008 and remained at Mystic Aquarium until the spring of 2009 while exhibit modifications occurred at the Shedd Aquarium. The transported belugas were initially placed into a holding pool physically separated from the three resident belugas (one male and two females approximately 27 yrs) at Mystic Aquarium. However, all belugas could establish visual and auditory contact with each other. Blood and saliva samples had already been collected and archived for a subset of the transported belugas at time points before, during and after the transport and introduction. Catecholamines will be measured in blood via High Performance Liquid Chromatography; adrenocorticocotrope hormone (ACTH), cortisol and aldosterone will be measured via established chemiluminescent and radioimmunoassays at the Animal Health Diagnostic Center, Endocrinology, College of Veterinary Medicine or via enzyme immunoassay (EIA) at the Mystic Aquarium; immune function including the ability of lymphocytes to proliferate, quantification of lymphocyte subsets and phagocytosis and respiratory burst of neutrophils and monocytes (via flow cytometry) will be assessed. Methodology for quantification of cortisol in beluga saliva will be worked out as well as determination of diurnal patterns if any in saliva. The relationships among hormones before after the stressors will be evaluated as well as the relationships between the hormones and immune function.

To fulfill objective 2, samples of blood, saliva, blow and feces will be collected at time points before, during and after out of water examinations for 2-3 aquarium resident whales. Catecholamines, ACTH, cortisol, aldosterone and immune function will be measured as above. Methodology for quantification of cortisol in beluga saliva and blow and corticosteroids in feces will be developed. The relationships among hormones before and after the stressors will be evaluated as well as the relationships between the hormones and immune function. The quantitative and temporal relationships of corticosteroid hormones across the different matrices will be evaluated.

Tracy Romano (P.I.) is primarily responsible for overseeing all sample collection and analyses, the development and transition of hormonal assays, and data integration and analyses. She will coordinate the project with the Co-Investigators both (on-site and off-site), she will write the results of the research in manuscript format for publication and present the research at scientific meetings and public forums.

Tracey Spoon (Co-P.I.) will play an activate role in sample collection, assay development and coordination of sample analyses in the laboratory, and work closely with the technician and graduate student on the analyses in the laboratory. She will work with the P.I. on data integration and analysis as well as publication.

Steve Lamb (Co-P.I.) will be responsible for the ACTH, aldosterone and cortisol assays that will be conducted at the AHDC at Cornell and advise and assist the P.I. and Co-P.I. with hormonal assay development and validation and transition of technology to Mystic Aquarium.

Laura Thompson, PhD student at the University of Connecticut (UConn), Department of Marine Biosciences will contribute to development of an assay for measuring stress hormones in cetacean blow as well as play a role in carrying out the immune function assays in the laboratory and assist with sample collection and data analyses.

WORK COMPLETED

Objective 1: Progress has been made on the archived samples in which belugas were sampled before, during and after transport and before, during and after novel introductions. Complete blood cell counts including total white blood cell (WBC) and differential counts along with phagocytosis and respiratory burst activity of granulocytes and monocytes were assayed immediately following blood collection. Additional samples were archived for subsequent analysis of catecholamines, cortisol, ACTH, aldosterone, lymphocyte proliferation and immunophenotyping. To date, hormone (cortisol, ACTH, and aldosterone) and catecholamine analysis (epinephrine, norepinephrine and dopamine) have been completed. Results on the influence of translocation and introduction to a novel environment on the innate immune system, as measured by phagocytic and respiratory burst activity has been published (Spoon and Romano, 2012).

Cells have been archived for assessing the influence of translocation and introduction to a novel environment on the adaptive immune system, as measured by lymphocyte proliferation and immunophenotyping of subpopulations of lymphocytes. Assays for immunophenotyping of lymphocyte subpopulations have been initiated and all samples from all translocated belugas and one resident beluga have been completed. Further, experiments have been initiated to determine the optimal parameters for the lymphocyte proliferation assay in belugas. It is expected that these results with the catecholamine and hormone data will be published in a similar manner as the previous manuscript focused on the innate response.

Objective 2: Behaviors for blood sampling, blow, saliva and feces collection are under continuous reinforcement on Aquarium belugas in order to fulfill objective 2. Monthly sampling and archiving has occurred for these tissue matrices on a routine basis. To date, one out of water event for 3 whales has been conducted.

Blood, blow, saliva and feces were taken at the same time points before, during and after the out of water event (stretched, removed from the water and placed on the deck for physical examination (approximately 30 minutes)). Complete blood cell counts along with phagocytosis and respiratory burst activity of granulocytes and monocytes were assayed immediately following blood collection. The remaining samples were archived for subsequent analysis of catecholamines, cortisol, ACTH, aldosterone, lymphocyte proliferation and immunophenotyping.

To date, archived samples have been analyzed for catecholamines and circulating concentrations of cortisol, ACTH, and aldosterone. Further, assays for immunophenotyping the lymphocyte subpopulations of archived samples are nearly complete and the initial experiments have been done to determine the optimal parameters for the lymphocyte proliferation assays in belugas. These parameters will be the same as those utilized for the lymphocyte proliferation assays conducted as part of Objective 1.

Since the last report period, five commercially available cortisol immunoassay kits were tested for use in quantifying cortisol in beluga blow and saliva samples (Salimetrics, MP Biomedical, Enzo Life Sciences, Caymen and Arbor Assays). A pool of blow, saliva and plasma samples from 4 beluga whales were used to test these specific matrices with the cortisol EIA kits. The final decision on which kit to use was based on the best results across all matrices. Additional tests were carried out on the chosen kit for intra- and inter- assay variability as well as interfering substances utilizing a charcoal stripping technique.

There were major challenges faced with the project over the past year. The Co-PI on the project, Dr. Tracey Spoon passed away unexpectedly in early May, 2012. In September, 2012 a research scientist was hired (Dr. Mandy Keogh) and will assume Dr. Spoon's role for the project. Also, with the first out of water event, phlebitis was observed in two of the three whales. This since has almost resolved, but has prevented additional out of water events from taking place. The veterinarian has approved moving forward and the out of water events are currently being scheduled for fall and winter. Due to these major setbacks and additional minor setbacks, a no cost extension through September 2013 has been proposed and granted for this effort.

RESULTS

Objective 1: Results on the influence of translocation and introduction to a novel environment on the innate immune system, as measured by phagocytic and respiratory burst activity has been published (Spoon and Romano, 2012).

Hematological analysis revealed significant differences in circulating lymphocyte counts for both the resident and translocated belugas. Specifically, resident belugas showed a decrease in circulating lymphocytes upon introduction of the translocated whales while the translocated belugas showed changes in circulating lymphocyte counts following transportation and introduction to a novel social environment. These findings are relevant as lymphocytes play a key role in the adaptive immune response. Currently we are continuing with assays designed at assessing the influence of translocation and introduction to a novel environment on the adaptive immune system. To date samples collected from all translocated belugas and one resident beluga has been completed for immunophenotyping of subpopulations of lymphocytes. Figure 1 displays the results from one resident beluga.

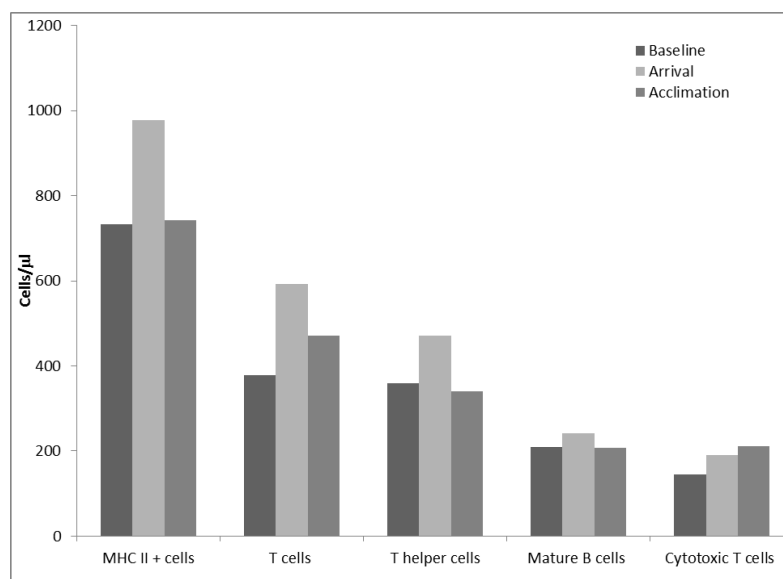


Figure 1. An example of the results from the immunophenotyping assays used to identify subpopulations of lymphocytes for one resident beluga before, during and after arrival of the Shedd belugas to the habitat.

Objective 2: The first out of water event included 3 resident belugas. Complete blood cell counts along with phagocytosis and respiratory burst activity of granulocytes and monocytes were assayed immediately following blood collection and statistical analysis of the results are currently underway. In regards to blood cells, whale one (female) showed increasing numbers of white blood cells and neutrophils from baseline after being out of the water 10 and 20 minutes and began to show a decrease after 30 min, while whale 2 (female) remained fairly constant until the post evaluation 24 hrs later, showing an increase in wbc, neutrophils and lymphocytes. The male remained relatively constant in wbc and neutrophils with a peak level after 20 min but showed a decrease in lymphocytes in the post samples 1 hr and 24 hrs later. The results for phagocytic activity, a key measure of innate immune function, from the first out of water experience for the male resident beluga is presented in Figure 2. Female one showed a decrease in neutrophil and monocyte phagocytosis and respiratory burst after 30 min on the beach and post 60 min. Values began to rise 24 hrs later and increased from baseline in the following post samples. Female two showed a decrease in phagocytosis and respiratory burst for neutrophils and monocytes after 30 min on the beach, while the male beluga increased in these measures.

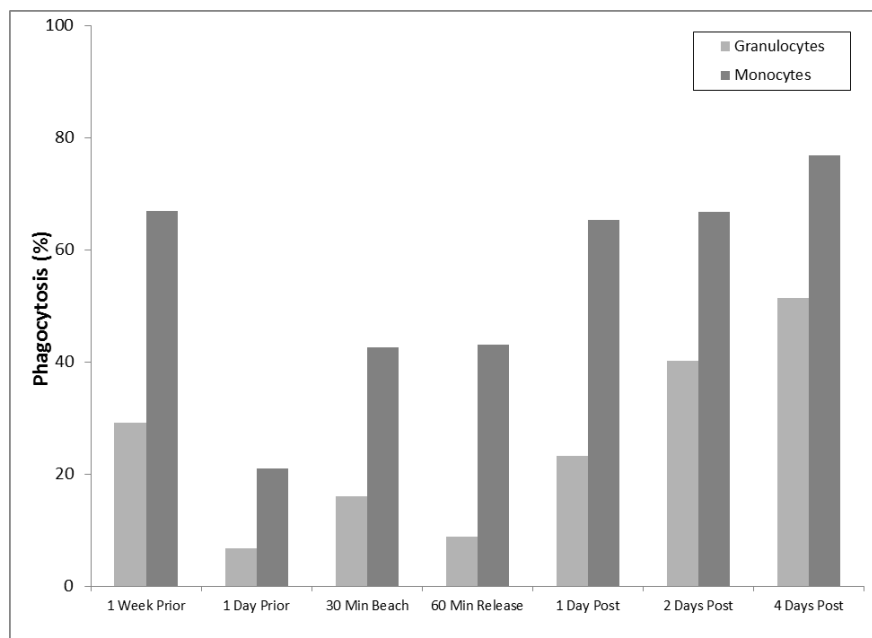


Figure 2. Phagocytosis in granulocytes and monocytes prior to, during and following the out of water event for one resident beluga.

Cells have been archived for assessing the influence of the out of water event on the adaptive immune system, as measured by lymphocyte proliferation assay and identification of subpopulations of lymphocytes. Immunophenotyping of lymphocyte subpopulations have been completed for all samples collected as part the first out of water event. Figure 3 presents the results from one beluga prior to, during and following the out of water event. For each time point, five subtype populations of lymphocytes were identified for all time points. The Lymphocyte proliferation assay will be completed following the determination of optimal parameters for the lymphocyte proliferation assays in belugas.

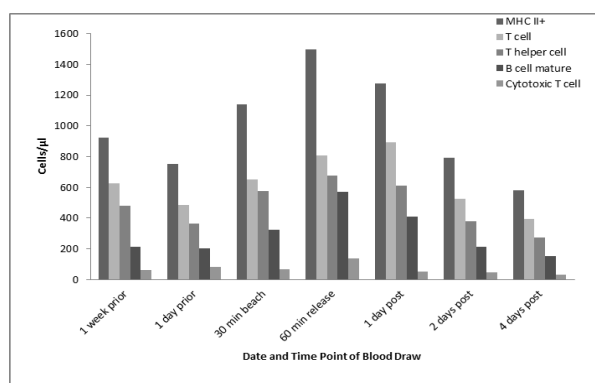


Figure 3. An example of the results from the immunophenotyping assays used to identify subpopulations of lymphocytes for one resident beluga prior to, during and following the first out of water experience.

In addition to completing the immunophenotyping, all catecholamine and hormone concentrations in blood from each time point have been quantified. During the first out of water experience ACTH was correlated to both cortisol ($r=0.870$) and norepinephrine ($R=0.469$). Only cortisol and ACTH were significantly elevated while the animals were beached out of water (Table 1).

Table 1. Mean, standard deviation and statistics for selected hormones and catecholamines collected from 3 belugas during the out of water event.

	Prior	Beach	Post	Repeated Measure ANOVA
Cortisol	2.4 ± 1.3	7.4 ± 1.9	2.0 ± 0.6	$F=24.96, p=0.006$
ACTH	11.1 ± 5.1	23.7 ± 5.3	10.2 ± 7.2	$F=74.941, p=0.001$
Norepinephrine	685.1 ± 139.3	628.7 ± 229.6	487.5 ± 139.6	$F=0.902, p=0.475$

Regarding the determination of matrix (blow, saliva, plasma) accuracy with the EIA kit, results from the standard with buffer were plotted against results from the standard with charcoal stripped blow (Figure 4), saliva or plasma. A best fit line was plotted and the standards spiked with the stripped blow should provide the same results as the normal standard providing a slope of 1 and intercept of zero. In this case, the slope was calculated at 0.9448. This less than 10% difference from a slope of 1 indicates that there may be some minor interference with the assay. The intercept was calculated at -18.746, indicating that all of the hormone was likely not removed from the pooled sample. This test will be repeated for blow as well as saliva and plasma. Intra- and inter- assay %CVs for the results obtained with blow were calculated to be 8.26 and 11.80 respectively, both of which fall within the acceptable range for recommended use of the assay. For saliva, intra- and inter- assay variability were calculated as 7.22 and 5.65 respectively while for plasma these were calculated as 9.06 and 12.27.

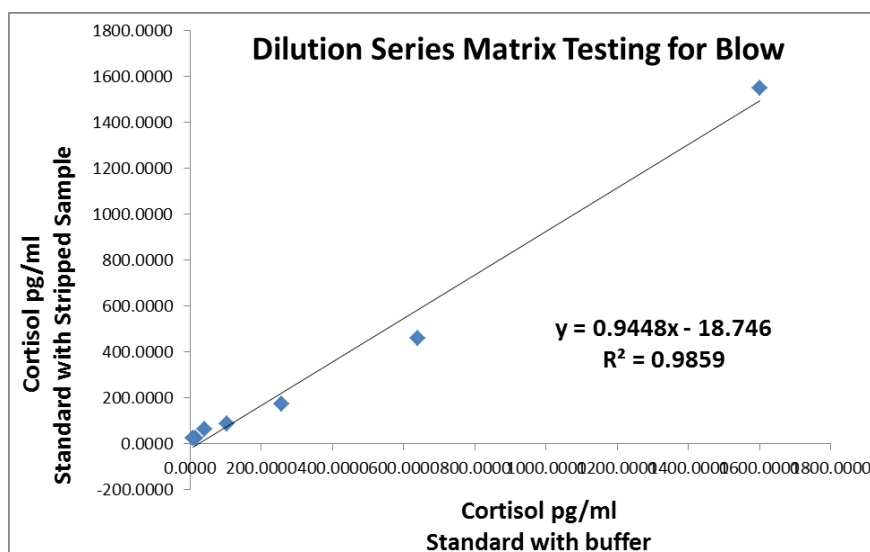


Figure 4. *Dilution series comparing kit standard with buffer to kit standard with charcoal stripped blow.*

IMPACT/APPLICATIONS

There is increasing concern regarding the potential effects of anthropogenic sound on marine mammals. The U.S. Navy is under continuous scrutiny with regards to sonar exercises and impacts on marine mammals. While studies have been conducted on behavioral and auditory responses in marine mammals with respect to anthropogenic sound there is a lack of scientific data and knowledge of the physiological impacts of loud sound exposure on marine mammals. There are many limitations and constraints in investigating the effects of anthropogenic sound as a “stressor” and impacts on the physiology of marine mammals. Despite these limitations there is a recognized need for such studies.

Investigation of the physiological response to stressors is very difficult in marine mammals given the difficulty in imposing stressors on marine mammals that will elicit a response, the lack of validated assays for measuring stress hormones, the difficulty in obtaining samples without causing stress, and obtaining a large enough sample size to draw significant conclusions. We are uniquely positioned at Mystic Aquarium, a division of Sea Research Foundation, Inc. to overcome the above obstacles and can provide a better understanding of the relationships among hormones in different matrices and in relation to immune function after stressor events. We can also define and compare the quantitative and temporal relationships of hormones across different matrices. This basic information is needed to lay the groundwork for understanding the impact of anthropogenic sound on marine mammals individually and at the population level.

RELATED PROJECTS

Title: Variability of Hormonal Stress Markers Collected from a Managed Dolphin Population

PI: Dorian Houser, PhD National Marine Mammal Foundation

Longitudinal study of a large dolphin population to characterize stress markers in different matrices.

Proposed effort also includes investigating the responsiveness of the thyroid and corticosteroid hormone production pathways.

Title: Pathophysiology of Stress in Wild and Managed-Care Bottlenose Dolphins (*Tursiops truncatus*)
PI: Pat Fair, PhD National Ocean Service
Study investigating hormones and immune function in wild dolphins vs. two different populations of managed-care dolphins (Aquarium setting vs. San Diego Bay).

Title: Baseline Health Measurements in Wild Belugas
PI: Tracy Romano, PhD Mystic Aquarium, a division of Sea Research Foundation
Study investigating hormones and immune function in wild beluga whales.

PUBLICATIONS

Spoon, T.R. and T.A. Romano. 2012. Neuroimmunological Response of Beluga Whales (*Delphinapterus leucas*) to Translocation and a Novel Social Environment. *Brain, Behavior and Immunity* 26:121-131.